AMRL-TR-68-132

BIOCHEMICAL PHARMACOLOGY OF HYDRAZINES TOXICITY

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MAY 1969

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AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO

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Foreword

This study was initiated by the Toxic Hazards Division of the Aerospace Medical Research Laboratory. The research was performed by the Institute of Chemical Biology, University of San Francisco, San Francisco, California 94117, under Contract No. AF 33(615)-3829. Kenneth C. Back, PhD, Chief, Toxicology Branch, was the contract monitor for the Aerospace Medical Research Laboratory. The work was performed in support of Project 6302, "Toxic Hazards of Propellants and Materials," Task 630202, "Pharmacology-Biochemistry," and Work Unit 019, "Research on the Biochemical Pharmacology of Hydrazines Toxicity." This study was started in March 1966 and was completed in February 1968.

The authors wish to express their appreciation to Mr. George Ledin, Jr., for his help in experimental design and statistical evaluation of data. The authors also thank Mr. Joseph Castagna, Jr., Miss Sandra Stratman, Miss Marion Doyle, and Mrs. Helen Graham for their laboratory assistance.

This technical report has been reviewed and is approved.

C. H. KRATOCHVIL, Colonel, USAF, MC
Commander
Aerospace Medical Research Laboratory

Abstract

The toxic action of 1,1-dimethylhydrazine (UDMH) and monomethylhydrazine (MMH) may be mediated by the inactivation of pyridoxal in the brain. One possibility considered was the formation of a hydrazone between the pyridoxals and the substituted hydrazine. Pyridoxal dependent enzymes were investigated. UDMH and MMH inhibited both glutamic acid decarboxylase and DOPA decarboxylase. Transaminases (amino transferases) which required α-ketoglutaric acid as a substrate were not affected by the hydrazines tested. Further work was conducted to refine an ultrasensitive bioassay method for the detection of each congener of the vitamin B_6 group. The microorganisms investigated for the assay were a neurospora and a yeast. Some indirect evidence was obtained which implies that UDMH injected intraperitoneally can be detected in the central nervous system. A mathematical model for hydrazines-induced convulsions was developed. It is now possible to predict the time lapse after administration of the convulsigen and the onset of seizure if only three data points are given.

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INTRODUCTION

The rocket fuels 1, 1-dimethylhydrazine (UHMH) and monomethylhydrazine (MMH) are convulsant agents. All species investigated responded similarly to these convulsigens (ref 1, 2, 3). Characteristic of the convulsion process after an intraperitoneal injection of these agents is a time lapse between their administration and the onset of the seizure. For a related substituted hydrazine, thiosemicarbazide (TSC), Jenney and Lee (ref 4) suggested a quantitative representation. A straight line was approximated when the $\log_{10}$ dose was plotted against the time lapse prior to the onset of the convulsion. This relationship had a limited applicability for it was linear only in a relatively small dose range. The dose-time relationships for UDMH and MMH were studied by O'Brien *et al.* (ref 5) for rats, and by Furst and Gustavson (ref 6) for mice. The latter group included data for the pyridoxal (and 5-phosphate) hydrazones.

The fact that the pyridoxal hydrazones are more toxic than the alkylhydrazines themselves (ref 6) suggests that the mechanism of action of the hydrazines involves brain pyridoxal. Indirect evidence for the involvement and perhaps inhibition of pyridoxal was given by Dubnick et al. (ref 7) who found that the pyridoxal antagonist 4-deoxypyridoxal exacerbates the toxicity of the hydrazine compound phenelzine. McCormick and Snell (ref 8) proposed a mechanism for thiosemicarbazide (TSC) toxicity; they postulated that the TSC combined with pyridoxal formed a hydrazone, which was a kinase inhibitor. In the presence of the inhibitor, pyridoxal could not be phosphorylated to pyridoxal-5-phosphate. No direct evidence was presented that TSC or the hydrazone crossed the blood-brain-barrier.

Pyridoxal-5-phosphate plays an important role in amino acid metabolism in the brain; it is a coenzyme for all nonoxidative transformations of the amino acids (ref 9, 10) such as decarboxylation, transamination, racemization, β-elimination and γ-elimination. Inactivation of this coenzyme by inhibition or by lowering of its absolute content in the rodent brain would be reflected in the decreased activity of the decarboxylases and transaminases. The enzymes are essential for the formation and metabolism of a number of brain biogenic amines like serotonin, norepinepherine, dopamine and GABA.

This project was undertaken to determine the biochemical mechanism of convulsant action of the alkylhydrazines, UDMH and MMH. The areas investigated were:

1. The change in pyridoxal content of rodent brain, before and after induced convulsion. This required developing an ultrasensitive detection method for the individual vitamin B_6 congeners in rodent brains.

2. The potential inhibition by the alkylhydrazines of decarboxylases in rodent brains.

3. The potential inhibition by the alkylhydrazines of some brain transaminases.

4. The detection of UDMH in rodent brain after intraperitoneal administration.

5. The development of a more exact mathematical expression of the relationship between the dose of the convulsigen and the time lapse before the onset of the seizure. The equation would hold over the entire dosage range and provide useful information for prospective studies.

Throughout this report, the following abbreviations are used for the hydrazine compounds:

1, 1-dimethylhydrazine (UDMH) and monomethylhydrazine (MMH). The congeners of the Vitamin B_6 group will be designated as: pyridoxine or pyridoxal (POL), pyridoxamine (PAMINE), pyridoxal (PAL). Their respective 5-phosphates will be indicated as in pyridoxal-5-phosphate (PALP). The hydrazones of PAL or PALP will be shown with a dash followed by the respective hydrazine as in: PAL-MMH, PALP-UDMH. Other abbreviations of chemical compounds are explained at the approximate place of their introduction in the text.

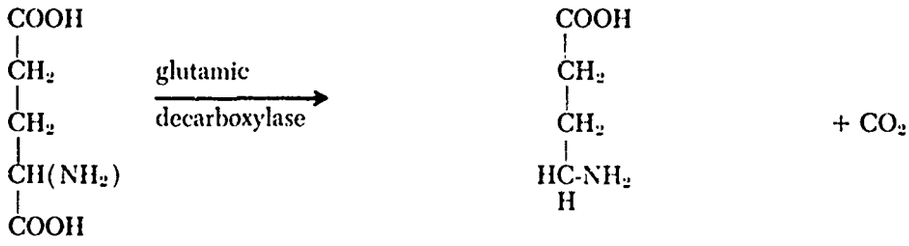
Section II

EFFECT OF UDMH, MMH AND THEIR B_6 HYDRAZONES ON THE ACTIVITY OF BRAIN DECARBOXYLASES

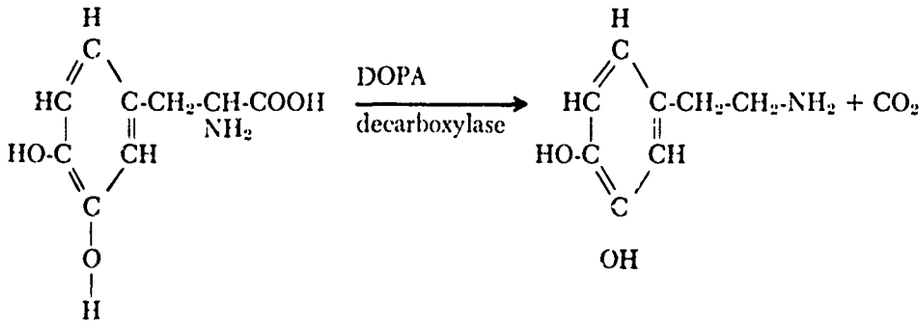
INTRODUCTION

The toxic mechanism of action of convulsant hydrazines UDMH and MMH may involve the inactivation or depletion of PALP in the central nervous system. As part of this program, a study was made of the inhibitory effect of these alkylhydrazines and their pyridoxal hydrazones on two decarboxylation enzymes, glutamic decarboxylase and DOPA decarboxylase. Both of the enzymes are pyridoxal-dependent.

The reactions are:



glutamic acid CABA



DOPA DOPamine

Enzyme activity can be measured by the amount of amine formed (ref 11), or by the CO_2 liberated. This study utilized ^{14}C carboxyl-labeled amino acids, the liberated $^{14}CO_2$ being trapped and counted in a liquid-scintillation counter. The reaction vessels were designed in the Institute of Chemical Biology, and the method used was that of de Ropp and Furst (ref 12).

MATERIALS AND METHODS

Male Swiss-albino mice (Berkeley Pacific Laboratories) weighing 18-20 g. were randomized, then injected intraperitoneally with a sufficient amount of an unbuffered saline solution of alkylhydrazine (UDMH: Eastman Organic Chemicals, MMH: Aldrich Chemical) or the PAL or PALP hydrazones (synthesized in our laboratory) to induce convulsions (ref 6). The following com-

pounds were used: MMH, UDMH, PAL-MMH, PAL-UDMH, PALP-UDMH, PALP-MMH. Mice injected with saline only were used as controls. Two mice were included in each group, and the experiments were replicated three to five times. Promptly after convulsion the mice were decapitated and, upon immediate removal their brains were weighed and homogenized with a teflon glass homogenizer in three volumes of 0.02 M ice-cold phosphate buffer at pH 7. The homogenate was placed in a dialysis bag (¼"-tubing) and dialyzed overnight against one liter of phosphate buffer at 4° C. The contents of the dialysis bag were rinsed into a graduated cylinder with buffer and the volume adjusted to give the equivalent of either 25 or 12.5 mg of tissue per ml.

Radioactive substrates used in these experiments were DL-3, 4-dihydroxyphenylalanine-1-^{14}C (New England Nuclear, NEN-245), with a specific activity of 2.36 mc/mM; and DL-glutamic-1-^{14}C acid (New England Nuclear CMMO22) specific activity 2.5 mc/mM. The labeled substrates were dissolved in sufficient phosphate buffer to make a solution containing 1 mg of the L-isomer per ml and stored frozen. For use, they were diluted with the corresponding cold amino acid always at a concentration of 1 mg of the L-isomer per ml. In practice, a solution giving about 600 CPM per μg of L-isomer proved satisfactory. The ten-to-fifteen-fold dilution of the hot substrate with cold was prepared 10 ml at a time and stored frozen.

PALP (Nutritional Biochemical Corp.) was dissolved in buffer to give a 1 mg/ml solution, diluted for use to give a final concentration of 1, 10 and 100 μg/ml. The cofactor was stored frozen but lost its activity in solution when kept longer than six weeks.

The hydrazines and hydrazones were dissolved in buffer and diluted for use to give final concentrations of 1×10^{-3}M, 10^{-4}M, and 10^{-5}M. They were subsequently found to be unstable in solution, a factor which introduced a variable in some of the earlier experiments. In later work, fresh solutions were made each day.

The enzyme reaction was carried out in the vessel shown in Figure 1. It consisted of two parts, the reaction chamber bearing the male joint and the absorption chamber bearing the female joint. Before use, the two halves were connected.

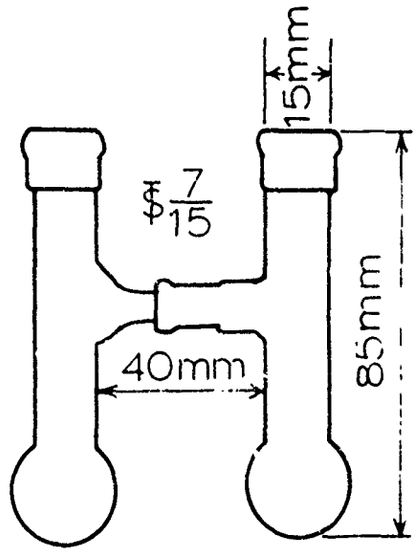


Figure 1. Enzyme Reaction Vessel

One ml of brain suspension was diluted to give the desired tissue concentration, then added to the reaction vessel containing PALP and the hydrazines or the hydrazones, when these were to be tested in vitro, to give a total volume of 1.9 ml. The two openings were sealed with serum caps (Aloe Scientific #V72400E); the air was replaced by nitrogen under reduced pressure by the insertion of a hypodermic needle through the cap which was attached by a three-way stopcock to a tank of nitrogen and a pump. The substrate (100 μg of the L-isomer in one ml of buffer) was added to the enzyme after 10 minutes preincubation on a Dubnoff shaking incubator at 37°C. Blanks were prepared by immersing the reaction vessels in a beaker of boiling water for 2 minutes to inactivate the enzyme before adding the substrate. Incubation time was 30 minutes. The reaction was stopped by injection of 0.1 ml 3N sulfuric acid through the serum cap into the reaction vessel. The absorbing vessel was injected with 0.4 ml of phenylethylamine in methanol (1:3). Then 0.1 ml of a saturated solution of cold sodium carbonate was added to the reaction vessel to flush out dissolved $^{14}CO_2$. The absorption vessel was disconnected and the phenylethylamine was rinsed quantitatively by means of a disposable pipette into a scintillation vial with 10 ml of scintillant solution (6 g PPO, 200 mg POPOP in 1 liter toluene). The vials were counted in a Packard Tri-Carb liquid scintillation spectrometer. The amount of decarboxylation was expressed as μg of L-isomer decarboxylated per gram of brain tissue per hour.

A series of in vitro experiments was performed. Brains from mice given no injection of convulsive agent were obtained, homogenized and prepared as previously described. The final tissue level in these experiments was 25 mg/ml. To test the possible inhibitory effect of UDMH, MMH, PAL-MMH and PALP-UDMH, these compounds were added directly to the reaction mixture at concentrations of 1×10^{-3}, 10^{-4}, 10^{-5}M and at 0. Also added was PALP at levels of 0, 1, 10, 100 μg/ml.

RESULTS

Glutamic Decarboxylase Activity of Brains from Convulsed Mice

In Table I, the glutamic decarboxylase activity of brain tissue from mice convulsed from various hydrazines and hydrazones is compared with that of control mice injected only with saline. The dialyzed brain homogenates still possessed decarboxylase activity even when no PALP was added. This activity was increased about four-fold in the controls when 1 μg/ml of PALP was added. A further small increase occurred with 10 μg/ml PALP, but when the level was raised to 100 μg/ml, the cofactor exerted a slight inhibitory effect.

All of the six compounds tested decreased the glutamic decarboxylase activity of the brains from convulsed mice. This lowering was greatest when no PALP was added to the reaction mixture. The most active compounds were MMH and UDMH, both producing an inhibition of about 70% in the absence of PALP. This inhibition could not be attributed to hydrazine passively carried over in the brain itself since all such material would have been removed by dialysis. The enzyme was inactivated and the inactivation was partially reversed by the addition of PALP.

The PAL and PALP hydrazones though inhibitory, were less so than the free hydrazines themselves.

TABLE I

GLUTAMIC DECARBOXYLASE ACTIVITY IN BRAINS OF MICE CONVULSED WITH HYDRAZINES COMPARED WITH THAT OF SALINE INJECTED CONTROLS

Values: μg L-glutamic acid decarboxylated per gram of tissue per hour

COMPOUND	No. of Exp.	PALP Concentration (μg/ml) 0	1	10	100
MMH	5	30	249	319	201
Saline Control		101	409	450	354
% Inhibition		70.3%	39.1%	29.1%	43.2%
UDMH	3	28	280	324	276
Saline Control		101	412	455	429
% Inhibition		72.3%	32.0%	28.8%	35.7%
PAL-MMH	3	64	312	324	240
Saline Control		108	392	394	372
% Inhibition		40.7%	20.4%	17.8%	35.5%
PAL-UDMH	3	69	280	284	224
Saline Control		98	382	395	296
% Inhibition		29.6%	26.7%	28.1%	24.3%
PALP-MMH	1	65	148	372	264
Saline Control		110	413	462	365
% Inhibition		40.9%	64.2%	19.5%	27.7%
PALP-UDMH	3	48	270	294	248
Saline Control		95	420	440	360
% Inhibition		49.5%	35.7%	33.2%	31.1%

DOPA Decarboxylase Activity of Brains from Convulsed Mice

In Table II, the DOPA decarboxylase activity of brain tissue from mice convulsed with various hydrazines and hydrazones is compared with that of saline-injected controls. MMH significantly reduced enzyme activity in these preparations but only in the absence of PALP. None of the other compounds affected significantly the activity of this enzyme under these experimental conditions.

Glutamic Decarboxylase Activity of Brain Homogenates Incubated in the Presence of Hydrazines and Hydrazones

Table III summarizes the effect of two hydrazines and their PAL hydrazones on glutamic decarboxylase of mouse brain when they were added directly to the reaction mixture.

Both MMH and UDMH inhibited the action of this enzyme at a concentration of 1×10^{-3}M, and the inhibition was not totally reversed by any of the levels of PALP added. At two lower levels of these substances (1×10^{-4} and 10^{-5}M), there was still some inhibition of the enzyme which was not reversed by PALP. The two hydrazones, however, were much less inhibitory and in some experiments, not inhibitory at all. It is possible that such inhibitory action as these compounds appeared to exert was due to breakdown products formed in solution.

TABLE II

DOPA DECARBOXYLASE ACTIVITY IN BRAINS OF CONVULSED MICE COMPARED WITH THAT OF SALINE INJECTED CONTROLS

Values; μg DOPA decarboxylated per gram of tissue per hour

COMPOUND	No. of Exp.	PALP Concentration (μg/ml) 0	1	10	100
MMH	2	60	200	255	165
Saline Control		121	402	465	600
% Inhibition		50.4%	50.2%	45.2%	72.5%
UDMH	2	137	350	497	575
Saline Control		115	410	487	685
% Inhibition		−19.1%	14.6%	−2.1%	16.1%
PAL-MMH	2	118	545	500	500
Saline Control		98	465	525	545
% Inhibition		−20.4%	−17.2%	4.8%	8.3%
PAL-UDMH	2	143	610	655	780
Saline Control		182	565	685	700
% Inhibition		21.4%	−8.0%	4.4%	−11.4%
PALP-MMH	2	120	575	582	545
Saline Control		121	562	570	650
% Inhibition		0.8%	−2.7%	−1.4%	16.2%
PALP-UDMH	2	136	550	585	457
Saline Con.		120	690	660	580
% Inhibition		−13.3%	20.3%	11.4%	21.2%

TABLE III

GLUTAMIC DECARBOXYLASE ACTIVITY (μg/g per hour) OF BRAIN HOMOGENATES INCUBATED IN THE PRESENCE OF HYDRAZINES AND HYDRAZONES

COMPOUND	No. of Exp.	Compound Concentration (M)	PALP Concentration (μg/ml) 0	1	10	100
MMH	2	0 M	110	420	460	352
		10^{-5} M	62	296	370	291
		10^{-4} M	38	280	370	286
		10^{-3} M	3.7	11	21	28
UDMH	2	0 M	115	436	471	385
		10^{-5} M	56	325	375	295
		10^{-4} M	41	291	360	261
		10^{-3} M	22	36	43	32
PAL-MMH	2	0 M	102	396	425	410
		10^{-5} M	98	400	385	426
		10^{-4} M	81	321	296	332
		10^{-3} M	78	192	277	275
PAL-UDMH	2	0 M	108	415	417	392
		10^{-5} M	110	426	431	385
		10^{-4} M	103	431	433	406
		10^{-3} M	101	436	450	402

DOPA Decarboxylase Activity of Brain Homogenates Incubated in the Presence of Hydrazines and Hydrazones

Table IV summarizes the effect of the four compounds on DOPA decarboxylase activity of mouse brain. In these experiments the compounds were again added directly to the reaction mixture. Both alkylhydrazines exerted an inhibitory action on this enzyme at all three levels used and the inhibition could not be reversed by addition of PALP. This enzyme was rather less sensitive to the inhibitory action of these substances than was glutamic decarboxylase. The two hydrazones of pyridoxal exerted a weak inhibitory action (about 20% inhibition at the highest concentration).

TABLE IV

DOPA DECARBOXYLASE ACTIVITY (μg/g per hour) OF BRAIN HOMOGENATES INCUBATED IN THE PRESENCE OF HYDRAZINES AND HYDRAZONES

COMPOUND	No. of Exp.	Compound Concentration (M)	PALP Concentration (μg/ml) 0	1	10	100
MMH	2	0 M	122	408	495	681
		10^{-5} M	131	310	445	415
		10^{-4} M	90	336	295	360
		10^{-3} M	42	102	133	103
UDMH	2	0 M	116	392	483	653
		10^{-5} M	75	278	385	397
		10^{-4} M	51	164	370	333
		10^{-3} M	50	54	122	228
PAL-MMH	2	0 M	120	420	492	660
		10^{-5} M	117	390	490	625
		10^{-4} M	121	340	425	545
		10^{-3} M	120	370	460	560
PAL-UDMH	2	0 M	125	410	470	670
		10^{-5} M	111	397	380	672
		10^{-4} M	131	394	425	595
		10^{-3} M	109	345	300	575

DISCUSSION AND CONCLUSIONS

None of the hydrazines or hydrazones tested could be classified as potent enzyme inhibitors when compared, for example, with α-hydrazino-methyl-DOPA, which gives about 50% inhibition at 1×10^{-6}M. The compounds however, significantly lower the activity of both glutamic and DOPA decarboxylase in brains of mice which had been convulsed. The fact that this inhibition could not be completely reversed simply by addition of PALP suggests that the removal of PALP by its combination with hydrazine is not the only mechanism by which it exerts its effect. Also, since convulsions can be induced by pyridoxal hydrazones confirms this view. *In vitro*, however, the combination of hydrazine with the cofactor probably is largely responsible for the inhibition. Under these conditions, the pyridoxal hydrazones are only weakly inhibitory or not inhibitory at all.

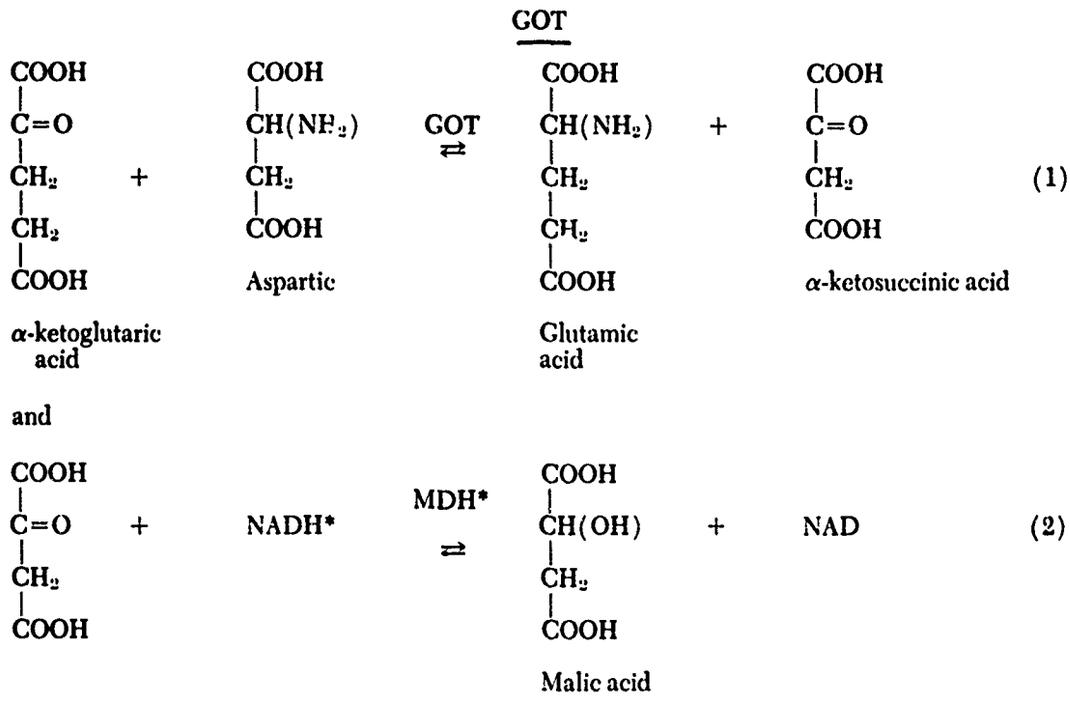
It must be borne in mind that the activity of these compounds in the body may be due to one of the breakdown products or a product of their interaction with body tissues. This possibility deserves further investigation.

Section III

THE EFFECT OF SOME HYDRAZINES AND HYDRAZONES ON BRAIN TRANSAMINASES

INTRODUCTION

Since transaminases (now called amino transferases) are also reported to be PALP dependent (ref 10), the possible inhibitory effects of the alkylhydrazines and their hydrazones was investigated. Selected for this study were two brain transaminases, glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT). Both reactions involve coupled enzyme systems. The equations are given for:



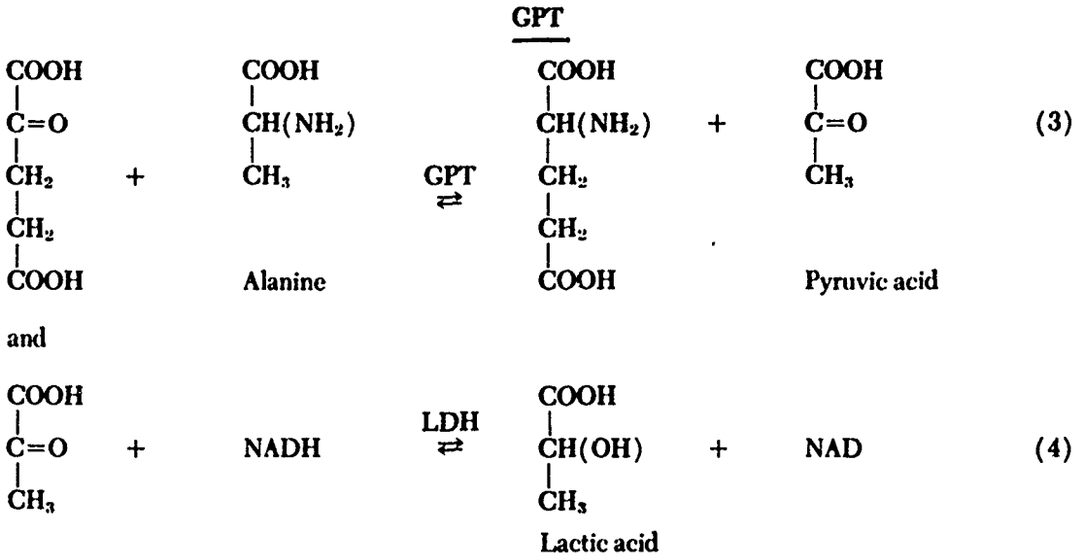
*The abbreviations used in these equations are:

MDH .....malic dehydrogenase

LDH.....lactic dehydrogenase

NADH..... reduced nicotinamide-adenine dinucleotide

NAD..... .....nicotinamide-adenine dinucleotide



The change: NADH ⟶ NAD in reactions (2) and (4) can readily be measured by determining the decrease in the optical density at 340 mμ for a given time period. The rate of change for this reading is related to activity of the respective enzyme.

This work was designed to show whether the six compounds under investigation (UDMH, MMH, PAL-UDMH, PAL-MMH, PALP-UDMH, PALP-MMH) would influence the rate of action of brain transaminases when added directly to the reaction mixtures at levels ranging from 1×10^{-3} to 1×10^{-6}M.

METHODS

Preparation of Enzyme

Adult male Swiss-albino mice were decapitated and the brains removed and weighed. Average brain weight was 420mg. Brains were homogenized in a teflon-glass homogenizer in four times their weight of borate buffer pH 8.2. This was further diluted to give one part tissue in fifty of homogenate. The preparation was dialyzed against borate buffer overnight. The GOT activity did not change when the dialyzed homogenate was stored frozen, but the activity of the GPT decreased rapidly. For this reason brain homogenates used for study of the latter enzymes were prepared from fresh brain. Before use, the homogenate was placed in an ultracentrifuge and spun at 100,000 x g for 30 minutes. The clear supernatant provided the source of the transaminase.

Assay Procedure

Kits (Worthington) prepared for the determination of GOT and GPT activity in serum were used in these experiments. Reagents in the kit were dissolved in 2.2 ml of water. The brain enzyme preparation was added to a cuvette in a volume of 0.5 ml and the hydrazines in 0.3 ml giving a reaction mixture final volume of 3.0 ml. Materials to be tested were added to the reaction mixture at concentrations of 1×10^{-6}, 1×10^{-5}, 1×10^{-4} and 1×10^{-3}M. A mixture to which buffer only was added provided the control; PALP was added to give final concentrations of 0, 1, 10 and 100 μg/ml.

Immediately upon mixing the solutions, the reaction was initiated, a stopwatch was started, and the optical density (OD) was read at one minute intervals. Results were expressed as average change in OD per minute.

RESULTS

Percentage inhibition of glutamic-pyruvic transaminase in mouse brain by MMH and UDMH is shown in table V (means of three estimates).

TABLE V

PERCENTAGE INHIBITION OF GLUTAMIC-PYRUVIC TRANSAMINASE IN THE MOUSE BRAIN BY MMH AND UDMH

	CONCENTRATION			
COMPOUND	OM	1×10^{-5}M	1×10^{-4}M	1×10^{-3}M
MMH	0%	4.9%	16.3%	70.1%
UDMH	0%	6.4%	15.7%	55.4%

The remaining compounds (PAL-MMH, PAL-UDMH, PAL-MMH, PALP-UDMH) were tested over a range from 1×10^{-6}M to 1×10^{-4}M but had no significant inhibitory effect on this enzyme.

Glutamic-oxaloacetic transaminase was not inhibited by MMH or UDMH over the range 1×10^{-5} to 1×10^{-3}M or by the four hydrazones over a range of 1×10^{-6} to 1×10^{-4}M. Addition of PALP to give concentrations of 1, 10 or 100 μg/ml did not affect the rate of the reaction.

DISCUSSION AND CONCLUSIONS

Although pyridoxal is reported to be the coenzyme in enzymatic transaminases, especially between α-ketoglutaric acid and a number of L-aliphatic amino acids, the vitamin does not seem to be inactivated by the alkylhydrazines except at very high concentrations. Since in vitro formation of the hydrazones is accomplished simply by mixing PALP, or PAL with either UDMH or MMH, it can be assumed that the hydrazones thus formed are not inhibitory as evidenced by their failure to modify the enzyme reactions.

The conclusion can be drawn that the mechanism of convulsant action of the alkylhydrazines does not involve these transaminases investigated. It is of interest that the rate of transaminase was not enhanced by the addition of exogenous PALP.

Section IV

DEVELOPMENT OF AN ULTRASENSITIVE QUANTITATIVE BIOASSAY METHOD FOR VITAMIN B_6 USING NEUROSPORA

INTRODUCTION

An assay method for the determination of vitamin B_6 content in mouse brains had to be developed before achieving the objectives of this project; i.e., to detect minute changes in PALP in brains just after convulsions induced by MMH or UDMH. It was anticipated that the initial B_6 levels per mouse brain would be in the nanogram range and that the change would have to be detected at the picogram levels. No chemical analysis is sensitive at these low levels, although a recently introduced gas chromatographic method may have promise (ref 13); but these procedures have not been applied to tissues.

Microbiological assays are unusually sensitive, and a method for the determination of total vitamin B_6 was developed by Atkin *et al* (ref 14) employing a yeast. This method was modified by Snell and Rannefeld (ref 15), and when a commercial medium was introduced more uniform results were obtained (ref 16). After the x-ray mutants of the fungus, Neurospora sitophila, became available it was found that one of the strains was vitamin B_6 dependent; this strain was then used for a bioassay of this vitamin (ref 17, 18).

The lower limit for detection of vitamin B_6 by these methods as reported in the literature was in the microgram range, values not sensitive enough for this work. Furthermore these procedures were not developed for the detection of individual congeners. A reproducible separation of the individual components of the vitamin B_6 group has already been achieved in these laboratories (ref 19) by the use of paper chromatography; the objective of this phase of the work was to develop an ultrasensitive bioassay method that could quantitatively determine PALP and PAL in the necessary nanogram or picogram range. Neurospora sitophila was the microorganism chosen for this project.

Many variables were investigated and attempts were made to find the optimum conditions for reproducible results with the bioassay method. Different experiments were conducted to determine: a) the best way to clean the glassware, b) if shaking the cultures had an effect on the growth rates, c) the temperature sensitivity of the neurospora, d) a method of preserving the inoculum through lyophilization to minimize loss of sensitivity of the fungus to PAL due to apparent back mutation, e) if the fungus was inhibited, or was growth supported by the PAL or PALP hydrazones, f) the optimum conditions for the assay.

METHODS

A. Preparation of the Glassware

The nature of the experimental work required a very meticulous procedure for laboratory glassware cleaning. The procedure consisted of the following steps:

(1) All glassware was washed with Alconox solution, then rinsed with distilled water.

(2) The glassware was soaked in a bath of diluted sodium dichromate solution (120 g $Na_2Cr_2O_7$ + 1600 ml concentrated sulfuric acid 2000 ml water).

(3) The glassware was rinsed ten times with distilled water and ten times with distilled-deionized water (d-d-w).

(4) Finally, the glassware was dried and covered to prevent contamination from the air.

This method was compared with a control method which consisted of washing the glassware with Alconox solution and rinsing with distilled water only (Step 1). The rates and total amount of growth cultures of Neurospora sitophila were compared by growing the organism in Basal Medium containing 100 nanograms PAL per flask; ten-125ml Erlenmeyer flasks were prepared the same way. Also, the extent of growth was estimated visually on a 0-5 scale. The mycelium was then harvested, dried in a desiccator and weighed to the nearest 0.1 mg.

B. Shake – Flasks

Comparisons were made on growth rates between continuous shaking of flasks and allowing the vessels to remain undisturbed at the same temperature.

C. Temperature Sensitivity of N. sitophila

The temperature sensitivity of N. sitophila was investigated as follows:

(1) A serial dilution with d-d-w was used to prepare a pyridoxal HCl solution of 100 picogram/ml from a standard solution. Sterilization was done by Seitz filtering.

(2) Erlenmeyer flasks (125 ml) with assay medium were prepared in triplicate. Controls contained 10 ml Basal Medium + 10 ml d-d-w and assay flasks had 10 ml Basal Medium + 9 ml d-d-w. All flasks with medium were autoclaved at 121°C for 20 minutes.

(3) To each assay flask was added 1 ml of pyridoxal solution. The final concentration was 5 picograms of PALP/ml of medium.

(4) Spores from three N. sitophila slant cultures were placed in suspension in 10 ml sterile d-d-w. The turbidity of this suspension was adjusted with sterile d-d-w to read 5% transmittance at 590 mμ in a Bausch and Lomb Spectronic-20 spectrophotometer. Each flask was inoculated with 0.2 ml of this mixture.

(5) The cultures were incubated on a Dubnoff shaker for 20 hours. The experiments were conducted at the following temperatures: 21, 23, 25, 26, 27 and 30°C.

(6) The mycelium was harvested by centrifuging the cultures at 33,000 rpm for 20 minutes. The pellet was then removed, blotted on filter paper and dried to constant weight in a vacuum desiccator over $CaCl_2$.

(7) The weights of the mycelial mats thus obtained were recorded.

D. Preservation of the Neurospora by Lyophilization

Mycelia of a one-week-old subculture of the Neurospora were transferred into a preservative medium (1% monosodium glutamate and 3% dextrose in 10% PVP). The mixture was injected into liquid nitrogen using a syringe with a 16-gauge needle. The resultant freeze-dried bead-like Neurospora was then transferred into flamed ampules; the transferring was carried out with the aid of a flamed forceps which was also dipped into liquid nitrogen to facilitate the procedure. One half of the ampules were put into a nitrogen atmosphere (by holding the open ampule under liquid nitrogen) and the other half remained in air. All ampules were then sealed with an acetylene torch.

The lyophilized supply was subcultured in slants made of Bacto Neurospora subculture media. A suspension made of the lyophilized Neurospora was made into an inoculum and tested with the bioassay of B_6. The bioassay was carried out in Petri dishes containing Bacto-pyridoxine

media and Difco Agar; the hardened media provided a firm surface for the support of strips of chromatographic paper (Whatman No. 4). Sterile paper strips were spotted with measured amount (1 mg, 10^{-1} mg and 10^{-2} mg) of PAL hydrochloride and then placed on these Petri dishes which were then incubated at 30°C for 5 days.

E. Will Hydrazones Support or Inhibit Growth of Neurospora?

Studies were made to see if PAL-UDMH or PALP-UDMH could be a source of vitamin B_6 for, or be inhibitory to the growth of N. sitophila.

A stock solution of PALP hydrochloride was made in d-d-w at a concentration of 10 nanograms/ml, and Seitz filtered. Stock solutions at a concentration of 1000 nanograms/ml were made of PAL-UDMH and PALP-UDMH. Experiments were conducted according to the following:

(1) Basal medium, 10 ml, was added to a series of 125 ml Erlenmeyer flasks. To some were added just 10 ml d-d-w; to others were added 9 ml d-d-w and 1 ml of PALP stock solution; to a third set was added 10 ml d-d-w, and 0.5 ml of stock PAL-UDMH.

(2) To a series of 10 ml Basal Medium in flasks were added 1 ml of stock PALP solution and increasing volumes of the PALP-UDMH stock solution, so that the concentration of PALP-UDMH ranged from 100 to 900 nanograms per flask. These experiments were repeated with PAL-UDMH.

(3) To a third group of flasks were added 10 ml Basal Medium, no PALP, but increasing amounts of PAL-UDMH, or PALP-UDMH as the only source of vitamin B_6. Concentrations of these hydrazones ranged from 100-800 nanogram/flask. Controls for this series were d-d-w alone, and PALP at a level of 10 nanogram/flask.

All groups were then treated the same. Each flask was inoculated with 0.2 ml of N. sitophila spore suspension originally made up to read 5% transmittance at 590μ. The flasks were plugged and incubated in a Dubnoff shaker for 20 hours at 27°C. The medium was then centrifuged, and mycelium pellets were removed, blotted, dried and weighed.

F. The Final Assay Procedure Developed

N. Sitophila (ATCC 9276) was grown on Neurospora sub-culture Agar plates at 25°C. A reserve supply of cultures was prepared by the lyophilization technique described to insure genetic integrity of the strain. A homogenous inoculum was available for each successive assay and a new tube was used for each new assay. (See Appendix for final lyophilization procedure.) Each inoculum was prepared by suspending spores from a two-day old subculture of the fungus in d-d-w, and this was diluted to a standard turbidity of 5% transmittance at 590 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer.

A standard solution of PAL hydrochloride was prepared and by serial dilution in d-d-w a stock solution was obtained with a concentration of PAL (not hydrochloride) 20 picograms/ml. This was sterilized by passing through a Seitz filter. Heat inactivated this vitamin.

Assay vessels were 125 ml Erlenmeyer flasks containing 10 ml of basal medium, these were sterilized by autoclaving at 121°C for 20 minutes. Sterile PAL solutions were added in an amount so that successive flasks contained 0 to 7 picograms of 1 picogram increments. Final dilution to 20 ml was completed using d-d-w.

These flasks were inoculated with 0.2 ml of a fresh N. sitophila spore suspension. The cultures were then suspended in a Dubnoff shaker and agitated in the water bath for 20 hours at 26°C.

Upon completion of the growth period, cultures were tested for purity and the mycelia were harvested by centrifuging the cultures in centrifuge tubes for 20 minutes at 33,000 rpm. The supernatant fluid was discarded and the mycelial pellet was removed, dried between two pieces of filter paper to remove excess moisture and then dried at constant weight over $CaCl_2$ in a vacuum desiccator for 12 hrs. The dry mats were weighed to nearest 0.1 mg.

The same procedure was used to assay POL and PAMINE, except that these members of the vitamin B_6 complex were prepared as a stock solution at 40 picograms/ml.

In order to test the applicability of the N. sitophila assay procedure for the detection of paper chromatographed vitamin B_6 congeners, the following method was employed:

PAL hydrochloride was spotted on 1 x 1¾ strip Watmann Paper #4 at a concentration of 5 μg/spot. Following the procedure of Gustavson et al (ref 19), the paper was developed in a pyridine:butanol:water solvent system. After drying, the paper was examined under ultraviolet light, the fluorescent spot was marked and cut out. The PAL was eluted by agitating the paper for 15 minutes with 10 ml of acidified Basal Medium (pH 4.4). The eluate was Seitz filtered and added to 10 ml sterile d-d-w in the assay flasks using sterile technique; strips spotted with saline acted as controls.

The flasks were then inoculated with the 0.2 ml of prepared spore suspension, incubated and the mycelia harvested, dried and weighed.

This procedure was repeated using PAL at the picogram level.

RESULTS

A. Detection of the vitamin B_6 by the neurospora was markedly facilitated with chromic acid-washed glassware. A comparison of the two methods (in visual and dry-weight observations) is given in table VI. It can be seen that the qualitative estimation for extent of growth was unreliable for this level of PAL.

TABLE VI

NEUROSPORA SITOPHILA GROWTH. COMPARISON OF THE TWO METHODS OF GLASSWARE CONTAMINATION

(Visual Ranking (5 best growth) and Dry Weight Determinations (mg))

FLASK NUMBER		1	2	3	4	5	6	7	8	9	10
Acid	VISUAL	5	5	4	5	5	5	3	4	3	5
Washed	DRY-WT.	67.5	57.0	37.5	66.5	54.0	60.5	24.0	33.0	11.0	20.5
Alconox	VISUAL	5	5	5	5	3	3	3	3	4	4
Washed	DRY-WT.	34.0	57.5	54.0	60.5	14.0	13.0	66.5	19.3	22.7	22.5

	Visual Average Ranking	Dry Weight Average Determination
Acid Washed	4.40	43.15 mg
Alconox Washed	4.00	36.40 mg

B. The method employing shaking gave much more uniform results than that without the mixing; therefore this step became an integral part of every procedure used.

C. The neurospora used for bioassay (Beadle 299) appears to be temperature sensitive. The optimum temperature range for growth in shake cultures appears to be 26-27°C. Above and below these temperatures growth is less and the culture's dependency on PAL is substantially decreased. Results of a typical experiment are given in table VII.

TABLE VII

EFFECT OF TEMPERATURE ON THE GROWTH OF N. SITOPHILA

Dry Weight in mg

Temp. (C)		21	23	25	26	27	30
Controls	1.	3.5	3.0	3.4	3.0	1.4	1.2
(no PAL)	2.	3.5	3.0	2.8	2.5	1.4	1.0
	3.	3.4	3.5	3.0	3.1	1.4	1.0
	av.	3.5	3.2	3.1	2.9	1.4	1.1
Assay	1.	3.5	3.4	3.1	3.4	2.4	1.3
5 picograms/ml	2.	3.7	3.0	3.4	3.4	2.6	1.3
PAL	3.	3.6	3.5	3.1	3.5	2.5	1.0
	av.	3.6	3.3	3.2	3.4	2.5	1.2
Difference							
Test-Controls		0.1	0.1	0.1	0.5	1.1	0.1

D. The lyophilized neurospora both in the nitrogen and air atmospheres could be subcultured easily. After 48 hours growth was noted and after 5 days sporulation occurred. In the preliminary assay using Petri dishes adequate growth was noted in three days. Visually it could easily be estimated that the growth could be qualitatively related at the mg level to the amount of PAL spotted on the paper.

E. The hydrazones were not a source of vitamin B_6 for the fungus. Evidently under the conditions of the experiment, neither the PAL-UDMH or the PALP-UDMH hydrolyzed to liberate the free vitamin. The weight of mycelium harvested in the first set of experiments was 2.4 mg when no PAL or hydrazones was added, and the same value when only hydrazone was present. For the PALP controls, some growth was obtained.

When a constant amount of PALP was added to the basal medium (10 nanograms/flask) and increasing amounts of PAL-UDMH (from 0-800 nanograms/flask) were added, no differences in mycelium weight were found from flask to flask.

No concentration of PAL-UDMH increased the dry weight of harvested mycelium, average

weights ranged from 2.5 to 2.8 mg which were the same as for the d-d-w control. At 900 and 1000 nanograms/flask of PALP-UDMH, the harvested mycelium weighed from 3.3-3.8 mg.

F. The harvested mycelia obtained from the growth of N. sitophila on 5μg PAL eluted from the chromatographed paper strips are summarized in table VIII. Slight growth was always noted from the controls.

TABLE VIII

DRY WEIGHT OF N. SITOPHILA FROM 5μg PAL ELUTED FROM PAPER

Flask No.	Test Culture	Flask No.	Controls
1	19.4	5	4.8
2	6.6	6	5.0
3	9.4	7	3.0
4	7.6	8	3.0
Av.	8.5		3.7

The values of mycelia obtained when PAL was used at the picogram levels are given in table IX.

TABLE IX

DETECTION AND ASSAY OF PICOGRAM AMOUNTS OF PAL

Dry weight mycelia in mg

Trial		0 p/ml	1 p/ml	5 p/ml	7 p/ml
I		2.0	2.0	4.5	4.5
		2.6	4.0	4.0	5.0
		2.5	2.5	4.0	5.0
	Ave.	2.3	2.8	4.1	4.8
II		4.5	3.2	7.5	6.6
		2.5	–	2.5	6.2
		2.8	2.5	3.3	4.5
	Ave.	3.2	2.8	4.4	5.8
III		1.3	1.5	3.0	3.5
		1.4	1.6	3.0	3.8
		1.4	1.6	3.0	4.0
	Ave.	1.4	1.6	3.0	3.8

POL and PAMINE values are not as reliable as PAL in the low picogram range.

DISCUSSION AND CONCLUSIONS

A bioassay method for the detection and estimation of individual members of the vitamin B_6 complex has been developed. Using the fungus, N. sitophila, PAL can be detected as low as 5-7 picograms/ml. The method depends upon growing the mycelium as shake cultures, harvesting the growth, and weighing the final mats.

All congeners of this vitamin can be separated by means of paper chromatography, eluted from the paper with Basal Medium and assayed. The method is more sensitive for PAL than POL or PAMINE.

Loss of sensitivity of the fungus to vitamin B_6 was noted on successive culturing on artificial media. A method was developed to preserve new cultures by lyophilization. It is assumed that the loss of dependence of the micro-organism on vitamin B_6 was due to back mutations.

The temperature dependence of N. sitophila was studied, and the range 26-27°C was found to be optimum for growth.

This fungus is not inhibited by PAL-UDMH or PAL-MMH, but at very high levels of PALP-UDMH, 1,000 nanograms/ml, some growth of the Neurospora is noted.

Section V

USE OF YEAST FOR BIOASSAY OF VITAMIN B_6

INTRODUCTION

Tests were run to establish whether a yeast system, S. carlsbergensis (culture 4228, Standard Brands, N. Y.), was suitable for detection of vitamin B_6 congeners in the nanogram and picogram ranges (ref 15, 16). This assay system was intended as a cross-check for the Neurospora method described in the previous section. Also, this yeast exhibited a greater sensitivity to PALP than Neurospora.

MATERIALS AND METHODS

Vitamin-free yeast base was obtained from Difco Laboratories, Baltimore, Md., and lyophilized cultures of S. carlsbergensis (ATCC 4228) were purchased from the American Type Culture Collection, Rockville, Md.

Experiments were conducted to find the optimum conditions for the assay. Growth was estimated by counting particles on a Coulter Counter, and by turbidometric measurements using two colorimeters; a Klett colorimeter, and a Bausch and Lomb Spectronic 20 spectrometer at 590 mμ. The latter instrument proved most satisfactory and was used for this section.

Standard solutions of PAL hydrochloride were prepared in concentrations of 50 nanograms/ml and 50 picograms/ml. The basal medium, Difco Vitamin-Free Yeast Base, was made by dissolving 16.79 g in 100 ml d-d-w. All solutions were Seitz-filtered and stored in sterile cotton-stoppered flask. (Filtration was used in place of heat sterilization because of the heat lability of PAL.)

The assay medium was prepared in sterile, cotton plugged 16 mm, culture tubes according to the following scheme:

Conc. of pyridoxal per 1 ml of final medium	Nanograms/ml				
	0	1	3	5	7
ml of sterile H_2O in tube	9	8.8	8.4	8.0	7.6
ml of sterile basal medium	1.0	1.0	1.0	1.0	1.0
ml of sterile PAL HCl standard solution (50 nano/ml) added	0	0.2	0.6	1.0	1.4

Tubes of assay media thus prepared were inoculated with 0.1 ml of a suspension of S. carlsbergensis obtained from suspending one loopful of growth at 26°C from a potato-dextrose agar slant culture in 10 ml of sterile d-d-w. To insure a consistent amount of inoculum in successive assays, the transmittance (%T) at 590 mμ of the yeast suspension was adjusted to 20 using sterile d-d-w.

Inoculated cultures in triplicate were incubated on a rapid shaker at 26°C for 4.5 days. The extent of growth was measured optically by a change in %T as compared to pure d-d-w which was used as a reference. Control flasks were used to test cultures for purity.

RESULTS

Table X gives an example of data from a typical experiment.

TABLE X

DETECTION AND ASSAY OF PAL IN THE NANOGRAM RANGE OF CONCENTRATION WITH SACCHAROMYCES CARLSBERGENSIS

% Transmittance at 590 mμ diluted 1:1 with d-d-w

	Nanograms/ml				
Tube	0	1	3	5	7
1	31.0	32.0	25.0	19.0	12.0
2	30.0	35.0	25.0	21.5	7.5
3	32.0	36.0	26.0	18.0	15.0
Average	31.0	34.3	25.3	19.5	11.5
Corrected averages for undiluted cultures:					
	15.5	17.15	12.6	9.75	5.75

To assay the vitamin B_6 group at the picogram level, certain modifications were made in these techniques (ref 20). These are:

1. The slant cultures were grown at 31°C rather than 26°C.
2. The assay was run at 30°C rather than 26°C.
3. The incubation period was shortened from 4.5 days to 3.
4. The inoculum (1 loopful of growth from a potato-dextrose agar slant culture in 10 ml d-d-w) was incubated on a shaker 1 hour at 28°C before use.
5. Tubes of the assay medium were inoculated with 0.2 ml of the yeast suspension rather than 0.1 ml.

The data of an experiment are given in table XI.

TABLE XI

PICOGRAM RANGE ASSAY OF PYRIDOXAL HCl USING S. CARLSBERGENSIS

% Transmittance at 590 mμ

	Picogram/ml				
Tube	0	1	3	5	7
1	19.7	13.1	21.3	17.9	12.0
2	38.5	41.5	17.0	12.2	15.0
3	broke	35.5	23.9	26.1	17.4
Average	29.1	30.0	20.7	18.7	11.1

DISCUSSION AND CONCLUSIONS

The lyophilized culture of Saccharomyces carlsbergensis obtained from ATCC was successfully subcultured. Since direct transfer of the lyophilized culture to potato-dextrose agar was as successful as the transfer to nutrient broth and then subsequent transfer to potato-dextrose agar, the intermediate step was abandoned.

Most of the problems of erratic growth such as contamination and sensitivity of detection were overcome and detection was greatly facilitated. The feasibility of several detection methods was explored. The observations obtained with one method were checked against similar data from other methods and were found in very good agreement.

The % transmittance data for the yeast bioassays were obtained on both a Klett Colorimeter and a Bausch and Lomb spectronic 20 spectrophotometer. When the readings with the Klett for yeast cultures were completed, a check against Coulter Counter counts was taken on the same cultures. Klett readings did not correlate well with the total cell population data obtained with the Coulter Counter. Hence the Klett Colorimeter was judged unreliable and its use was discontinued. The Bausch and Lomb Spectrophotometer was found to be a more reliable instrument. The constant refining of the techniques of the yeast bioassay procedure produced the desired result of obtaining pyridoxal sensitivity of the organism in the picogram range of concentration. Though more work on this method is indicated, the procedure has been used to assay PALP in individual mouse brains.

Section VI

APPLICATION OF ULTRASENSITIVE QUANTITATIVE BIOASSAY METHODS FOR THE DETECTION OF VITAMIN B_6 IN RODENT BRAINS

INTRODUCTION

The ultra-sensitive bioassay methods, although not perfected, were found to be useful and were applied for the detection of Vitamin B_6 congeners in the brain. However, for this section only PAL and PALP were investigated. The method used involved paper chromatography to separate the components, elution of the individual components from the strips, and finally the bioassay procedures described in the two previous sections. Concentrations standards were in the picogram range.

This section consists of two parts:

A. The analysis of PAL and PALP in mouse brains.

B. The effect of PALP-UDMH on growth of Neurospora.

METHODS

Part A

For both sections A and B, mouse brains were prepared for chromatography by the following procedure:

(1) Two groups of mice were used. The first group was injected I.P. at a dose level of 500 mg/Kg of UDMH in saline. The control group was injected with a comparable volume of normal saline.

(2) After death by convulsions in the experimental group or cervical fracture in the control group, the entire brains were immediately removed, quickly frozen in liquid nitrogen, and weighed.

(3) Five mouse brains from each group were pooled and 1 ml of pyridine was added for every gram of brain tissue. Each mixture was homogenized for one minute, using an ultrasonic probe. (Bio-Sonic Instrument). Samples were kept cold at all times in a bath of acetone and dry ice.

(4) The homogenates were centrifuged for 5 minutes at 2500 rpm.

The supernatant from the centrifuged homogenate was chromatographed on Whatman Paper #4 by the procedure established by Gustavson et al (ref 19). Brain supernates and standards were diluted 10X before spotting on chromatography strips (0.1 ml of homogenate supernatant and 0.9 ml of a pyridine:butanol:water in ratio of 1.2:saturated).

The zones ("spots") of pyridoxal were cut from the chromatographed strips and the PAL was eluted in 10 ml Basal Medium. These areas were determined by control R_f values.

Various methods of elution were tested and the best results were obtained when a combination of the following procedures were used:

(1) To avoid the destruction of PAL by light, the strips were chromatographed and the "spots" were cut out in the dark.

(2) Basal medium was used for elution but the pH was adjusted to 5.0 so that PAL would be recovered more efficiently.

(3) A slight increase of temperature increased the recovery of PAL by elution, therefore this step was done in an incubator at 37°C without light.

(4) The beakers were intermittently agitated.

(5) The elution process was carried out for at least one hour to obtain maximum recovery of PAL.

(6) Two millipore filtration systems (Swinney Adapters with syringe and needle attachments) were introduced in place of the Seitz. This allowed separation of the experimental and control eluates. A Seitz filter was used to sterilize the known assay solution.

(7) To the sterile eluates in assay flasks were added 10 ml aliquots of sterile d-d-w.

(8) Each flask was inoculated with 0.2 ml N. sitophila spore suspension and the cultures were incubated at 27°C for 20 hours in a Dubnoff shaker.

(9) The mycelium was harvested and the dry weights recorded.

For PALP detection and quantitative assay, the same procedure was used, but the eluting solvent was 1 ml vitamin-free yeast base plus 9 ml d-d-w. The flask was inoculated with S. carlsbergensis.

Part B

The assay procedure followed that given in Part A, with the following modifications: instead of eluting PAL or PALP from the paper strips, solutions of PALP-UDMH of known concentration were added to 10 ml of assay medium. To a series of flasks was added 1 ml of a solution containing 1 μg/ml of PALP. To each flask was also added PALP-UDMH in increasing concentration from 100 to 1000 nanograms/flask.

RESULTS

Part A

The data from the determination of PAL in rodent brains is given in table XII. The weight of dry mycelium was converted to picograms PAL/ml of brain homogenate. Three separate trials are recorded.

Part B

From the results given in table XIII, it can be noted that PALP-UDMH was not an inhibitor of the neurospora used. The slightly greater dryweights of mycelium noted at the higher concentrations of the hydrazone were not statistically significant.

TABLE XII

DETECTION AND ASSAY OF PAL ISOLATED FROM RODENT BRAINS BY PAPER CHROMATOGRAPHY AND ASSAYED BY N. SITOPHILA

REFERENCE SOLUTIONS

TRIAL	FLASK	Picograms/ml 0	2	4	6	8	Control Brain	UDMH Convulsed Brain
I	1	1.03	2.03	2.22	3.05	2.62	2.60	3.39
	2	1.60	1.80	2.60	2.31	2.94	2.10	4.34
	Ave.	1.31	1.91	2.41	2.68	2.78	2.35	3.86
II	1	0.86	1.15	1.30	1.74	1.66	1.48	1.21
	2	0.90	1.09	1.35	1.48	2.03	1.16	1.71
	Ave.	0.88	1.12	1.32	1.61	1.84	1.32	1.46
III	1	2.06	2.40	1.90	2.27	2.27	2.84	2.47
	2	1.93	2.45	2.37	2.18	2.31	2.01	3.19
	Ave.	1.99	2.42	2.13	2.22	2.26	2.42	2.83
		OVERALL AVERAGES						
		1.39	1.83	1.95	2.17	2.29	2.03	2.71

TABLE XIII

PALP-UDMH INHIBITION STUDY

Flask Number	1	2	3	4	5	6	7	8	9	10
Nanograms PAL-UDMH/Flask	100	200	300	400	500	600	700	800	900	1000
Dry Weight Mycelium	2.49	2.69	3.37	3.46	2.93	3.40	3.25	3.08	3.80	3.25

DISCUSSION AND CONCLUSIONS

Part A

All experiments have consistently shown a greater PAL content in brains from mice convulsed by UDMH than in the normal controls. The order of magnitude averages 2 picograms greater. This small difference is not statistically significant. However, since there is no real decrease in the PAL content of convulsed brain, the decrease in B_6 dependent enzyme activity cannot be due to the simple depletion of PAL. Inactivation of PAL by hydrazone formation is still possible, and no evidence that supports or detracts from the theory of McCormick and Snell (ref 8) can be found in the experiments.

Part B

The addition of PALP-UDMH did not inhibit the growth of N. sitophila when PALP was present in the medium. The level of 1 μg PALP was high enough to give adequate growth. The hydrazone apparently was not a source of PALP for the neurospora.

Section VII

EVIDENCE THAT INJECTED UDMH IS FOUND IN THE CNS

INTRODUCTION

Indirect evidence is presented that UDMH may cross the blood-brain barrier after intraperitoneal administration. Until now, most theories of convulsive action of UDMH and MMH assumed that these alkylhydrazines did penetrate the brain barrier and exerted their action in the CNS. No supporting data were presented by others.

In brief, the circumstantial evidence is: following UDMH induced convulsions in mice, the brains were removed, prepared as previously described but PALP was added to the supernatant prior to chromatography. Examination of the paper strip revealed a new fluorescent spot which is not present in the supernatant of control brains. The chromatographic R_f value indicated the new compound to be PALP-UDMH.

MATERIALS AND METHODS

These animals were convulsed with UDMH, and the brains were removed and prepared as described in Section VI, Methods; Part A. The supernatants were spotted on Whatman #4 paper and chromatographed in the dark using pyridine:butanol:water as described in Gustavson, et al (ref 19). After the paper strips were dried, they were examined under ultraviolet light.

The experiments were repeated but this time a known quantity of PALP was added to the homogenates prior to centrifugation. These supernates were again chromatographed and examined for fluorescence.

In subsequent experiments, PAL, PAL-UDMH, PALP-UDMH were chromatographed singly and in all combinations. These compounds were also added individually to brains from UDMH convulsed mice and to controls. These brains were prepared, supernatants were spotted on paper and chromatographed.

The paper strips were exposed to ammonium hydroxide vapors, and the R_f values were measured for all fluorescent spots.

RESULTS

No fluorescent spots were noted on the paper strips chromatographed from the convulsed or control brain preparations. When the experiments were repeated but with PALP added to the homogenates, the ultraviolet light revealed a new "dark spot" fluorescence in convulsed brain preparations, but not in controls. The fluorescent spot was enhanced when exposed to ammonium hydroxide vapors. The R_f of the "dark spot" was identical to that of PALP-UDMH.

When PALP-UDMH was added to control or convulsed brain, or was added to the solvent system, and each preparation was chromatographed, the same R_f value was obtained for the "dark spot." All spots reacted similarly to ammonium hydroxide vapors.

A mixture of PAL-UDMH and PALP-UDMH was chromatographed, and separation was easily achieved, and the expected R_f values were found. After exposure to ammonium hydroxide vapors, and after examination under ultraviolet light, only PALP-UDMH gave the characteristic "dark spot."

DISCUSSION AND CONCLUSIONS

All of the evidence points to the fact that the "dark spot" is PALP-UDMH. Under ultraviolet light, free UDMH does not fluoresce; the hydrazone has a marked fluorescence. It is assumed that the hydrazone is easily formed in brain tissue. The evidence comes from the rapidity with which the compound is made by simply mixing the reactants in aqueous solution.

To date this appears to be the first evidence that UDMH does appear in the rodent brain after an intraperitoneal administration of the hydrazine agent.

Section VIII

DEVELOPMENT OF A MATHEMATICAL MODEL FOR THE CONVULSION PROCESS INDUCED BY HYDRAZINES

Its short duration makes the convulsion process one of the most difficult toxicological processes to study. The symptoms preceding the actual seizure are varied, and induced convulsions do not have exact pre-established patterns, although some signs occur with greater frequency than others. Precision equipment for measuring these signs was not available, and all symptomatic characteristics were observed without their more fine-detailed descriptions.

Each time a mouse convulsed, a certain selection of the most frequent symptoms – mydriasis, miosis, exophthalmos, enophthalmos, astasia, trismus, piloerection, catalepsy, lethargy, grand mal, and the more or less definitively final conditions of a sequence of petit mals followed by opisthotonos – was observed. The mathematical model had to reflect the severity of these symptoms as a function of the administered dose, and also provide an accurate description of the time lapse prior to the onset of the seizure.

The first series of convulsion experiments produced the necessary data on the basis of which the proposed model (ref 21) was written. With the aid of several Fortran IV computer programs it was possible to obtain values of time lapse (min.) over a very wide range of dose (mg/Kg). The surprising agreement between the experimental data points and the computed values led us to work out a more refined mathematical model incorporating a most valuable feature – its prospective (predictive) capability. All consequent convulsion experiments were therefore shortened to obtaining a few baseline points, or threshold values, and then for each convulsigen a computerized table of expected lag times was obtained. The success of this approach made it possible to economize in many respects especially in the number of rodents to be expended and thus cut down on laboratory time.

In essence the model utilized in this work is described in its elementary form by Ledin *et al* (ref 21). The successful application of this novel approach warranted a paper by Furst *et al* (ref 22).

If the data from a convulsion experiment (lag time vs dose of convulsant are plotted on regular coordinate paper with the dose on the abscissa, a hyperboloid curve is obtained; this curve is of the hyperbolic-exponential type, as best least-squares linearization is achieved only after inverse-ruling and logarithmic transformations. A simple logarithmic transformation (log dose vs time) is insufficient as it provides linearization only over a small dose range. The convulsion equation is then a member of the family

$$T \quad K\, e^{|p(D)/q(D)|}$$

where T is the time lapse prior to convulsion or death, D is drug dose, $p(D)/q(D)$ is a polynomial ratio of negative degree (the generalized polynomial hyperbolicity property), e is the base of natural logarithms ($e \quad 2.71828\ldots$) and K is a constant to be determined from experimental information. The equation used in our initial prediction trials with the computer was

$$T \quad T_0 + \frac{c_0}{(D\text{-}D_0)} + \frac{c_1(D\text{-}D_1)}{(D\text{-}D_0)^2} + \frac{c_2(D\text{-}D_1)(D\text{-}D_2)}{(D\text{-}D_0)^3} + \ldots$$

where D_0 is the "minimal dose" (that is, minimum dose for which convulsions take place and also the highest dose which does not induce convulsions) and the doses D_1 and D_2 are two suitably chosen experimental doses. The time T_0 is the minimal time of response (that is, the lag time corresponding to the maximum dose). The coefficients $c_0, c_1, c_2, \ldots$ were computed from these initial experimental points. A sequence of predicted times were obtained by substituting a monotonically increasing sequence of doses D at increments of 50 mg/Kg, and these times were found in excelient correspondence with the experimentally observed values. This equation, with new constants, was tested for each substituted alkylhydrazine and hydrazone utilized in this study.

The inverse of the area under each convulsion or death curve (from D_0 to a value D) of a particular convulsigen turned out to provide a measure of the intensity of the specific symptoms resulting from the administration of the agent. These estimates, a new concept in the literature are best coined as "convulsivility" (convulsant ability) and "lethality," and their usefulness in establishing a new basis for ranking a group of chemical agents is exemplified in table XIV where the potency is given as a ratio with UDMH taken as the arbitrary unit. These estimates also give an indirect judgment of potency or a conditional comparison of effectiveness, for they can be related to various therapeutic indexes and fit in very well with accepted criterions of mortality or poisonous efficacy.

TABLE XIV

Relative Potency

(Trapezoidal Estimates)

	HYDRAZINES			HYDRAZONES			
	UDMH	MMH	Hydrazine Anhydrous	PAL-UDMH	PAL-MMH	PALP-UDMH	PALP-MMH
"Convulsivility"	1.00	6.24	6.95	23.13	19.58	16.71	18.26
"Lethality"	1.00	7.73	5.92	18.82	17.40	21.39	17.44

Appendix

PREPARATION OF N. SITOPHILA FOR BIOASSAY

BASAL MEDIUM

Sucrose	15.0 g
Ammonium Tartrate	5.0 g
Citric Acid ($C_6H_8O_7H_2O$)	2.0 g
KH_2PO_4	2.5 g
$MgSO_4, 7H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
$FeCl_3$	5.0 mg
$ZnSO_4 \cdot 7H_2O$	2.0 mg
Biotin Solution	40.0 ml (4.0 μg)
Add water to	500.0 ml

LYOPHILIZATION MEDIA: Mist-desiccants

1 Part any Nutrient Broth

3 Parts fresh Rabbit or Sheep Serum

7.5% Glucose

or

30% any Nutrient Broth

1 Part Glucose

3 Parts Serum

PROCEDURE:

1. Seitz filter above media and disperse in 2 ml amounts into sterile constricted ampules. Store at −20°C until use.

2. Place a piece of culture with adherent agar in liquid Mist-desicans and then place in a deep freezer or alcohol-CO_2 bath.

3. Lyophilize tubes of cultures in Vertis freeze-dryer

4. Place in vacuum desiccator over P_2O_5 for 4 to 5 days.

5. When desiccator is opened use nitrogen which is passed through a tube of Drierite.

6. Seal the ampules with an oxygen flame.

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Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1 ORIGINATING ACTIVITY (Corporate author)
Institute of Chemical Biology
University of San Francisco
San Francisco, California 94117

2a. REPORT SECURITY CLASSIFICATION
UNCLASSIFIED

2b. GROUP
N/A

3 REPORT TITLE

BIOCHEMICAL PHARMACOLOGY OF HYDRAZINES TOXICITY

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)
Final Report, March 1966-February 1968

5 AUTHOR(S) (First name, middle initial, last name)
Arthur Furst
Waldemar R. Gustavson
Robert S. deRopp

6 REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
May 1969	32	22

8a CONTRACT OR GRANT NO	9a. ORIGINATOR'S REPORT NUMBER(S)
AF 33(615)-3829	
b. PROJECT NO 6302	
c. Task No. 630202	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d. Work Unit No. 630202019	AMRL-TR-68-132

10 DISTRIBUTION STATEMENT
This document has been approved for public release and sale; its distribution is unlimited.

11 SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Aerospace Medical Research Laboratory, Aerospace Medical Div., Air Force Systems Command, Wright-Patterson AFB, OH 45433

13 ABSTRACT

The toxic action of 1,1-dimethylhydrazine (UDMH) and monomethylhydrazine (MMH) may be mediated by the inactivation of phridoxal in the brain. One possibility considered was the formation of a hydrazone between the pyridoxals and the substituted hydrazine. Pyridoxal dependent enzymes were investigated. UDMH and MMH inhibited both glutamic acid decarboxylase and DOPA decarboxylase. Transaminases (amino transferases) which required alpha-ketoglutaric acid as a substrate were not affected by the hydrazines tested. Further work was conducted to refine an ultrasensitive bioassay method for the detection of each congener of the vitamin B6 group. The microorganisms investigated for the assay were a neurospora and a yeast. Some indirect evidence was obtained which implies that UDMH injected intraperitoneally can be detected in the central nervous system. A mathematical model for hydrazines-induced convulsions was developed. It is now possible to predict the time lapse after administration of the convulsigen and the onset of seizure if only three data points are given.

DD FORM 1 NOV 65 1473

Security Classification

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Hydrazines						
1,1-Dimethylhydrazine (UDMH)						
Monomethylhydrazine (MMH)						
Pyridoxine						
Vitamin B_6						
Pharmacology						
Biochemistry						
Toxicology						
Bioassay Methods						
Rodents						